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Host defense peptide expression in human cervical cells and regulation by 1,25-dihydroxyvitamin D3 in the presence of cytokines and bacterial endotoxin.

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Abstract

Host defense peptides (HDPs) in the pregnant female reproductive tract provide protection against infection. The relationship between HDPs and infection/inflammation is poorly understood. Therefore we investigated the regulation of HDPs by 1α, 25-dihydroxyvitamin D3 (1,25-(OH)2) in the presence/absence of infectious/inflammatory agents. Endocervical epithelial cells (END1/E6E7, n=6) were exposed to 1,25-(OH)2, calcipotriol, IL-1β, GM-GSF and LPS. Elafin, human beta defensin (hBD2), cathelicidin, secretory leucocyte protease inhibitor (SLPI), interleukin-8, 1,25-(OH)2 receptor and toll like receptor 4 (TLR4) expression was determined using qPCR and/or ELISA. HDP gene and protein expression was assessed in cervico-vaginal cells/fluid respectively from first trimester pregnant women (n=8-12). IL-1β, induced elafin and hBD2. 1,25-(OH)2-induced cathelicidin expression in the presence of IL-1β and LPS. 1,25-(OH)2 also attenuated IL-1β-induced IL-8 expression and LPS enhancement of TLR4. HDPs and TLR4 profiles in cervico-vaginal cells and fluid samples from pregnant women were similar to END1/E6E7 cells. In conclusion, HDPs are differentially regulated in END1/E6E7 cells. 1,25-(OH)2 induction of cathelicidin and suppression of IL-8 highlights a mechanism by which 1,25-(OH)2 supplementation could enhance the pregnant innate immune defenses.

Keywords

Host defence peptide, human cervix, inflammation, infection, 1,25-(OH)2.

Introduction

Spontaneous preterm birth (sPTB, particularly at gestations <34 weeks of pregnancy), is often associated with reproductive tract inflammation and ascending infection [1-3]. Several pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and TNFα, have been detected in cervico-vaginal fluid in women at high risk of sPTB and women in threatened preterm labour[3-5].

There is growing literature demonstrating the expression of host defence peptides (HDPs) in the human female reproductive tract, including the cervical mucus plug, [6-8]. Moreover, there is emerging evidence that suggests that the reproductive tract production of elafin is altered in bacterial vaginosis (BV) [7], chorioamnionitis [9] and in women at high risk of preterm labour [8]. Regulation of reproductive tract HDPs expression in pregnancies associated with spontaneous preterm birth is less well described.

Evidence from *in vitro* cell studies and the non-pregnant literature on HIV, indicates that HDPs are produced from epithelial cells and leucocytes in response to pathogens and damage associated molecular patterns (PAMPS and DAMPs) [10, 11]. Pro-inflammatory cytokines, neutrophil proteases, microbial endotoxins [7, 12] steroids [13, 14] and vitamin D [12, 15-17] have been shown to upregulate a variety of HDPs *in vitro*.

The potential interaction between vitamin D and HDPs is of interest, as vitamin D serum status has been shown to have an impact on female reproductive health [18, 19] and deficiencies have been linked with preterm birth [20, 21]. Indeed in a recent study, the majority of women at high risk of sPTB in our population had reduced or deficient plasma vitamin D concentrations[8].

The impact of vitamin D on cervical epithelial cell HDPs responses in the presence of inflammation has not been ascertained. Therefore the aim of this study was to investigate the

effects of the active form of vitamin D (1,25-(OH)2) , converted from 25-hydroxy1,25-(OH)2 by the intracellular enzyme CYP27B1) on HDP expression [elafin, human beta defensin (hBD2), cathelicidin and secretory leucocyte protease inhibitor (SLPI)] using an endocervical epithelial cell line (END1/E6E7) and to determine whether the response would be modulated in presence of inflammation (IL-1β, GM-CSF shown to be raised in women at risk of sPTB[3]) or endotoxin (LPS). We hypothesised that 1,25-(OH)2 in tandem with modulating HDPs would lead to suppression of inflammation. In addition, we sought to comprehensively describe HDP profiles (mRNA and protein) from freshly obtained endocervical and high vaginal cells (ECCs and HVCs respectively) from pregnant women in their first trimester of pregnancy.

Materials and Methods

Human recombinant interleukin-1 beta (IL-1β) and GM-CSF were purchased from R&D Systems Europe Ltd, Abingdon, UK. 1,25-(OH)2 and Lipopolysaccharide (LPS) from Escherichia coli 0127:B8 were purchased from Sigma-Aldrich, UK. Calcipotriol was purchased from Cayman Chemical, Cambridge Bioscience Ltd, UK. Endocervical cells (END1/E6E7) were purchased from ATCC, LGC Standards, Middlesex, UK. Keratinocyte serum-free medium was purchased from Invitrogen Life Technologies, Paisley, UK. Human elafin, Secretory leukocyte peptidase inhibitor (SLPI) and hCAP18/LL37 ELISA kits, elafin monoclonal antibody (clone TRAB20) and hCAP/LL37 18 monoclonal antibody (1-1C12 clone) were purchased from Hycult Biotech, Uden, The Netherlands. Human beta defensin-2 (hBD2) ELISA kit was purchased from Phoenix Pharmaceuticals Inc, Germany.

High Vaginal and Endocervical fluid and cell collection

Ethical approval from was obtained from City and East London Local Research Ethics Committee (REC reference: 13/LO/0393). All participants gave informed written consent for the collection of samples used in this study.

High vaginal fluid (HVF, n=12) and endocervical fluid (ECF, n=10) were obtained from low risk pregnant women in their first trimester of pregnancy (mean gestation: 12.1 ± 0.1 weeks.). Of the 12 HVF samples collected, 3 women had clinically graded bacterial vaginosis (BV). Of the 10 ECF samples collected, 3 women also had clinically graded BV. HVF was collected by inserting sterile speculum into the vagina to facilitate collection of Dacron swabs from the posterior fornix (10 seconds). ECF were then obtained by rotating a cytobrush within the cervical os (10 seconds). Dacron swabs/cytobrushes were then placed into ice-cold PBS (+ protease inhibitors), rotated for 30 s to release cells and centrifuged. HVF/ECF was removed and stored at -80°C. Remaining high vaginal cell (HVC) and endocervical cell (ECC) pellets were stored at -80°C.

Cell culture

END1 E6/E7 cells were cultured in keratinocyte serum-free (KSF) medium (n=6 individual passages). Growth media was supplemented with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract and 0.4 mM calcium chloride and replaced every 2 days. Cells were grown to approximately 80% confluence in 75 cm^2 culture flasks (Corning ®, Appleton Woods, Birmingham, UK) when they were either sub-cultured into 75 cm^2 culture flasks or plated into 6-well culture plates in 3 ml KSF growth medium. Cells were maintained at 37°C in 5% CO_2 and 95% air in a humidified atmosphere. Prior to experimentation, cells were growth factor-deprived and incubated in KSF treatment media (supplemented with 0.1% BSA and 0.4mM calcium chloride) for 24 h.

RNA extraction and qPCR

RNA was extracted from cultured uterine END1 E6/E7 cells, HVCs and ECCs using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. RNA concentrations obtained from HVS cell pellets was in the range of 55 – 120 ng/µl. RNA

concentrations obtained from cytobrushings were in the range of 40 – 249 ng/µl. Complementary deoxyribonucleic acid (cDNA) was synthesized using Quantitect (Qiagen, UK). Real-time polymerase chain reaction (PCR) was carried out using SYBR Green chemistry (Bioline) on a RotorGene 6000 (Qiagen, UK) using the primers as listed in Table 1. A pre-PCR cycle was run for 10 min at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 50 s followed by a final extension at 72°C for 15 s. Test samples were run in duplicate in parallel with cDNA standards of known gene copy number abundance (10^8 to 10^1 copies). Cycle threshold (CT) values were used for analysis, and abundance data for test samples were quantified based on the standard curve. Data for the genes of interest were then expressed relative glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was the most stable housekeeper from a panel of 3 (GAPDH, β-actin and β-2 microglobulin).

Enzyme-Linked Immunosorbent Assays (ELISAs)

Protein concentrations of elafin, SLPI, hBD2 and hCAP18/LL37 in cell culture supernatants were measured by ELISA according to the manufacturer's instructions.

Statistical Analysis

GraphPad Prism software (Version 6.07) was used for data analysis and graphical representation. Gene expression data (copy number) were normalised to GAPDH copy number from the same experiment and then analysed using the Kruskal–Wallis one-way analysis of variance followed by Mann-Whitney U test for individual comparisons. Cell culture supernatant protein expression data was normalised to total whole cell protein before being analysed as previously described. Data are expressed as median and interquartile range (box) ± range (whiskers) or mean ± SEM. $P<0.05$ was accepted as significant.

Results

Figure 1A-H illustrates basal expression of elafin, SLPI, hBD2 and cathelicidin in END1 E6/E7 cells. The most abundant HDP protein was SLPI, with a ranked expression profile in END1/E6E7 cells under resting condition of SLPI> elafin>cathelicidin>hBD2, this correlated with mRNA expression profiles. IL-1β (10 ng/ml) significantly induced mRNA expression of elafin and HDB2 mRNA at 24 h ($P<0.01$ for both) and translated to increased concentrations of secreted proteins into the cell culture medium ($P<0.05$ and $P<0.01$ respectively). Increased elafin and hBD2 protein expression was also detected after 24 hours exposure to IL-1β (data not shown). Neither cathelicidin nor SLPI mRNA or cell culture protein expression were altered by IL-1β.

In contrast, 1,25-(OH)2 treatment only induced expression of cathelicidin mRNA (6 hours, $P< 0.05$) and protein (24 h, $p<0.01$). Calcipotriol had less impact than 1,25-(OH)2, but there was a small significant increase in cell supernatant cathelicidin expression after 48 hours of treatment ($P<0.01$) despite a limited effect on mRNA at 6 hours.

The interactions between a pro-inflammatory environment on 1,25-(OH)2 and calcipotriol treatment are also illustrated in Figure 1 A-H. Neither 1,25-(OH)2 nor calcipotriol altered the magnitude of induction of elafin or hBD2 by IL-1β. Similarly, 1,25-(OH)2 induction of cathelicidin was not reduced in the present of IL-1β. The minimal effect of calcipotriol on cathelicidin protein expression was lost when added to cells pre-exposed to IL-1β. SLPI mRNA and protein expression was unaffected by any combination of treatment with a maintained cell culture medium concentration of approximately 100000 pg/mg of total cell protein seen for all conditions.

Similar experiments shown in Figure 2A-H determined whether HDP gene expression could be modified in the presence of endotoxin (LPS) and the cytokine GM-CSF. Elafin mRNA and

protein expression was significantly increased following 24-hour LPS treatment ($P<0.01$) (Figure 2A-B) but 1,25-(OH)2 treatment (6 h and 24 h) did not enhance or suppress this effect.

Similarly, LPS significantly enhanced hBD2 mRNA and protein expression ($P<0.01$), with no further change following 1,25-(OH)2 treatment (Figure 2C-D). GM-CSF, unlike IL-1β and LPS, had no effect on elafin or HBD2 mRNA or protein expression.

LPS pre-incubation had little impact on cathelicidin expression, but 1,25-(OH)2 still increased cathelicidin mRNA and protein post LPS treatment (Figure 2E-F, $P<0.01$ and $p<0.05$ respectively). GM-CSF had no effect on cathelicidin mRNA or protein expression. Similar to IL-1β and 1,25-(OH)2, neither LPS nor GM-CSF altered SLPI mRNA or protein expression (Figure 2G-H).

Both IL-1β and LPS significantly enhanced IL-8 mRNA expression after 24 hours (Figure 3A-B, $P<0.05$ and $P<0.001$ respectively). 1,25-(OH)2 and calcipotriol challenge 6 hours post IL-1β incubation was able to suppress this increase in IL-8 to basal levels ($P<0.01$ and $P<0.001$ respectively). LPS was also able to elicit a significant increase in IL-8 mRNA expression ($P<0.05$). 1,25-(OH)2 also appeared to suppress this induction, but this did not achieve statistical significance. Interestingly, LPS also induced TLR4 mRNA expression ($P<0.05$) which was significantly inhibited in the presence of 1,25-(OH)2 (Figure 3C, $P<0.05$). IL-1β had no effect on TLR4 mRNA expression either in the presence or absence of 1,25-(OH)2 or calcipotriol (Figure 3D). 1,25-(OH)2 receptor mRNA expression in END1/E6E7 was unaffected by any of the treatments (Figure 3E-F).

The relevance of these findings to native cervical epithelial cells was examined in cell pellets obtain using a cytobrush (ECCs) and high vaginal swabs (HVCs). There was no significant difference in HDP gene expression between the two sampling sites. The order of mRNA expression in ECCs was SLPI > cathelicidin > hBD2 > elafin and in HVCs was SLPI > hBD2

> cathelicidin > elafin (Figure 4A). Interestingly, the order of protein expression was SLPI> elafin> cathelicidin> hBD2 for both HVF and ECF, with cathelicidin protein expression in ECF significantly higher than HVF ($P<0.05$) (Figure 4B). Cathelicidin protein was only detectable in 8 out of 12 HVF and 7 out of 10 ECF samples analysed.

HVCs and ECCs expressed both the 1,25-(OH)2 receptor (VDR) and TLR4 mRNA at similar levels (Figure 4C).

Discussion

We have compared, for the first time, the expression and cell secretion profiles of four host defense peptides by a human endocervical epithelial cell line (END1 E6/E7) to ECCs and HVCs and ECF and HVF obtained from pregnant women. *In vitro*, HDPs were differentially regulated by cytokines, endotoxin and 1,25-(OH)2 in END1/E6E7 cells, with the influence of 1,25-(OH)2 treatment on cathelicidin expression retained in the presence of inflammatory cytokines or endotoxin. 1,25-(OH)2 also suppressed IL-8 and TLR4 expression.

In unstimulated END1 E6/E7 cells, SLPI and elafin were the most abundant HDPs; mRNA expression for both cathelicidin and hBD2 were relatively low, but secreted cathelicidin protein in the cell media was higher than hBD2. The mRNA profiles were mirrored in the cervical and high vaginal swab cell samples obtained from pregnant women which suggests the END1 E6/E7 model are useful for studying HDPs in relation to pregnancy.

Elafin and hBD2 were significantly induced following treatment with the pro-inflammatory cytokine IL-1β and LPS. These data are consistent with previous reports of hBD2, SLPI and elafin expression in other epithelial cell types [22-24]. It contrasts with a previous study by Stock and colleagues[7] which reported that END1 E6/E7 cell elafin mRNA was unresponsive to a 24

h challenge with IL-1β and that expression was only significantly up-regulated by LPS. Here we have a larger sample size and clearly show IL-1β induction of mRNA and protein expression after 24 h exposure to treatment which may account these differences.

SLPI basal expression was high and not altered by IL-1β/LPS which suggests that SLPI is constitutively expressed as a frontline immune defense. The lack of evidence of modulation by cytokines and 1,25-(OH)2 does not necessarily mean that it cannot be regulated, indeed SLPI concentrations have been reported to be greater in vaginal fluid from pregnant women (12 – 20 weeks gestation) compared to their non-pregnant counterparts, regardless of BV status [25], suggesting it may be induced by other agents such as placental steroids [26-28].

Elafin/hBD2, although inducible by IL-1β and LPS were not responsive to 1,25-(OH)2 and conversely, cathelicidin was unaffected by IL-1β and LPS but induced by 1,25-(OH)2. This complements a smaller study by Frew and colleagues [12], that just assessed the effect of 1,25-(OH)2 on cathelicidin mRNA in END/E6E7 and ECT/E6E7 cells. The ability of 1,25-(OH)2 to induce cathelicidin was retained even under inflammatory conditions, i.e. in the presence of IL-1β and LPS. It has been shown that cathelicidin has the ability to neutralise LPS using a murine macrophage cell line and a murine endotoxin shock model [29]. Thus, our data raises the possibility of 1,25-(OH)2 supplementation and/or the use of cathelicidin as a potential therapeutic agent in the treatment of bacterial infections or for those women at high risk of early sPTB. The involvement of other TLR receptors and ligands in modifying the cathelicidin response in END cells would be of interest given the potential influence of polymicrobial infections in sPTB [30], and reports of TLR2 activation and 1,25-(OH)2 induction of cathelicidn in macrophages. [17, 31-33]

In addition to the induction of cathelicidin, 1,25-(OH)2 treatment in the presence of LPS and IL-1β was associated with suppression of inflammatory mediator IL-8. LPS induction of TLR4

mRNA expression was decreased without affecting induction of other HDPs. A recent study by Gonzalez-Curiel *et al* [34] showed that calcitriol was able to suppress LPS-induced IL-1β and hBD2 gene expression without altering TGF-β, ANG and LL-37 gene expression *in vitro*. Similar reports of 1,25-(OH)2 attenuation of the production of pro-inflammatory mediators have been described in corneal epithelial cells and endometriotic stromal cells respectively [35-37]. This differential impact of 1,25-(OH)2 on cathelicidin has potential to be exploited clinically, albeit the concentrations used *in vitro* (based on concentration-response curve data) in our study are higher than those found circulating *in vivo*. However, the observation that LPS/IL-1β in presence or absence of 1,25-(OH)2 (10^{-6} M) had no effect on VDR mRNA expression indicates that the pathway was not down regulated. The active form of 1,25-(OH)2 used in this study would be available *in vivo* following intracellular conversion via the enzyme CYP27B1 and local concentrations are likely to be higher than those found in blood. The active form of 1,25-(OH)2 can affect many cell types including epithelial cells and has many cellular targets and biological effects [16, 38, 39].

In the present study, we also report that cathelicidin and elafin mRNA are expressed in freshly obtained ECCs. To our knowledge this is the first report of cathelicidin expression in freshly isolated ECCs from first trimester, low risk pregnancies which adds to a previous report of HDP expression in term pregnant cervical biopsies from primagravid women [7]. Our finding that elafin is also expressed in ECCs is in agreement with a recent report by Itaoka and colleagues [40] in which they identified elafin and SLPI protein in cervical cells (squamous and glandular) obtained from pregnant women throughout gestation.

HDP expression in HVF and ECF was very similar to that found in END1/E6E7 cell culture medium. Interestingly, cathelicidin protein was significantly higher in ECF than HVF. We

previously reported high cathelicidin concentrations in women at risk of preterm birth [8]. Elevated levels of cathelicidin in cervico-vaginal secretions have also been reported in BV [12].

The finding that cathelicidin was not detectable by ELISA in all samples assessed was similar to the study by Frew *et al* [12] which detected cathelicidin in only 60.8% of cervico-vaginal secretions analysed. Moreover, cathelicidin was undetectable in vulvar biopsies taken from women infected with human papilloma virus as well as healthy controls in a study by Erhart *et al* [41]. This suggests that cathelicidin expression may be individual- specific and not solely dependent upon inflammatory status.

To complement our previous study that reported raised elafin in cervico-vaginal fluid of women with a high risk of having a sPTB [8], we have now demonstrated that elafin mRNA and protein is detectable in freshly isolated ECCs and ECF both from women with and without evidence of clinically graded bacterial vaginosis. Our data is consistent with that of -Itaoka *et al* in which elafin mRNA was detected in cervical cells obtained from women throughout gestation and was elevated in women who had a preterm delivery compared to matched controls [40]. A limitation of our study is that we did not characterise the cells in our ECC and HVC samples, but Itaoka and colleagues reported that the cellular components of the cervical swabs obtained were mostly squamous cells and glandular cells in the ratio of 4:1. It would be useful in future experiments to assess the functional impact of HVF and ECF with different concentrations of HDPs in terms of using bacterial killing assays. Moreover, there are published studies which demonstrate the antibacterial properties of both elafin and cathelicidn [42, 43].

Elafin has been identified as a factor in non-pregnant female genital secretions, correlating with resistance to HIV infection, most likely a result of direct interaction between HIV-1 and elafin [44, 45]. This highlights the theoretical potential use of HDPs for therapeutic purposes in the female reproductive tract and sPTB, but this is a nascent field. Elafin has been shown to prevent intestinal inflammation in mouse models of colitis [46] with cathelicidin, elafin and SLPI also

modulating colitis when delivered intravenously or intracolonically in mouse colitis models [47]. More recently, cathelicidin has recently been shown inhibit H. pylori growth, destroy the bacteria biofilm, and induce morphological alterations in helicobacter pylori membrane [43].

The effects of 1,25-(OH)2 deficiency and its influence on female reproduction and pregnancy is well documented [18, 19]. However, currently there is no consensus on optimal 1,25-(OH)2 levels in pregnancy and the impact of supplementation on maternal outcomes and fetal development [48]. The differential regulation of cathelicidin and suppression of IL-8 in our study by 1,25-(OH)2 indicates another potential mechanism by which beneficial effects in pregnancy might be achieved by supplementation.

In summary, we demonstrate the presence and order of abundance of cathelicidin elafin, hBD2 and SLPI in high vaginal and endocervical cells and fluid from pregnant women in their first trimester of pregnancy. We have also shown that although a TLR4 ligand and inflammatory agents (LPS/IL-1β/GM-CSF) have no direct effect on cathelicidin production by endocervical cells, cathelicidin can be upregulated by 1,25-(OH)2 in an inflammatory environment and via this also suppress inflammation. Future studies will focus on elucidating the role of HDPs in prevention of infection/inflammation in both pregnant and non-pregnant female reproductive tract and will also address the potentials involvement of other TLR receptors e.g. TRL2) and their ligands.

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Table 1. qPCR primer sequences for amplification of human mRNA.

GENE	FORWARD	REVERSE	PRODUCT SIZE (bp)
hBD2	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA	89
Cathelicidin	TCGGTAGCTAACCTCTAC	ACAGGCTTTGGCGTGTCT	80
Elafin	TCGTGGTGGTGTTCCTCAT	ACGGCCTTTGACAGTGTCTT	92
GAPDH	GGAAGCTTGTCATCAATGGAA	TGGACTCCACGACGTACTCA	102
IL-8	GCCTTCCTGATTTCTGCAGC	CGCAGTGTGGTCCACTCTCA	151
TLR4	CCTCCCCTTCTCAACCAAGA	GCTCTGATATGCCCCATCTTC	148
VDR	AAGCTGAACTTGCATGAGGAG	GTCCTGGATGGCCTCAATC	108

Figure legends

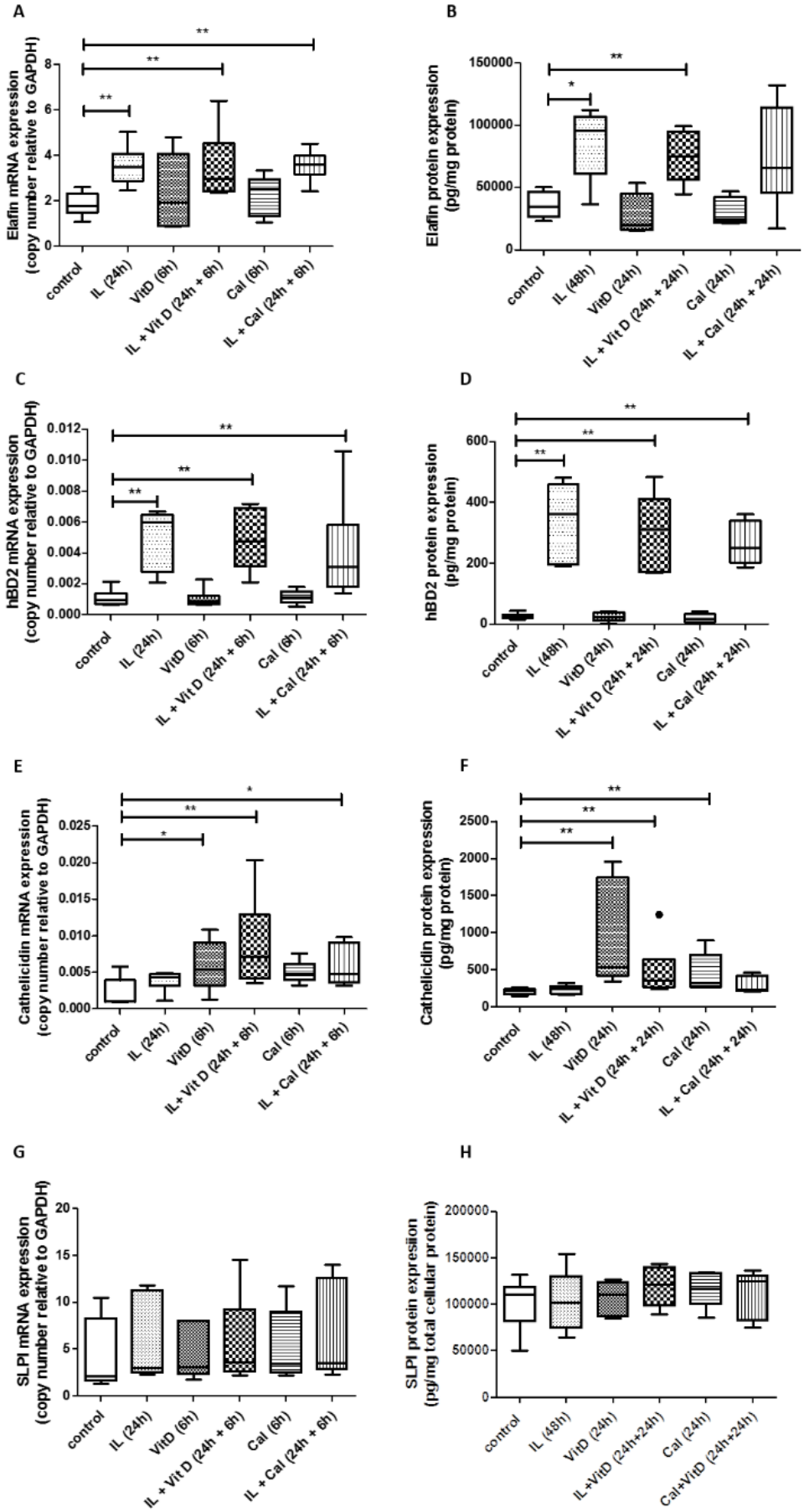
Figure 1. Effect of IL-1β (IL), 1α,25-dihydroxy1,25-(OH)2_3 (VitD) and Calcipotriol (Cal) on host defence peptide mRNA and protein expression in END1/E6E7 cells. Cells were pre-treated with IL-1β (10ng/ml) for 24 h and then for a further 6 h ± VitD/Cal (1μM) or with VitD/Cal (1μM) alone for the last 6 h of the experiment. Whole cell lysates were assessed for elafin (A), hBD2 (C), cathelicidin (E) and SLPI (G) mRNA expression. In addition, cells were pre-treated with IL-1β (10ng/ml) for 24 h and then for a further 24 h ± VitD/Cal (1μM) or with VitD/Cal (1μM) alone for the last 24 h of the experiment. Cell culture supernatants were assessed for elafin (B), hBD2 (D), cathelicidin (F) and SLPI (H) protein expression. Data are expressed as median natural antimicrobial peptide normalised copy number ± interquartile range (box) and range (whiskers). n=6. *P<0.05; **P<0.01 compared to control.

Figure 2. Effect of 1α,25-dihydroxy1,25-(OH)2_3 (VitD), Lipopolysaccharide (LPS) and GM-CSF on host defence peptide mRNA and protein expression in END1/E6E7 cells. Cells were pre-treated with LPS (10μg/ml) or GM-CSF (10 ng/ml) for 24 h and then for a further 6 h ± VitD (1μM) or with VitD (1μM) alone for the last 6 h of the experiment. Whole cell lysates were assessed for elafin (A), hBD2 (C), cathelicidin (E) and SLPI (G) mRNA expression. In addition, cells were pre-treated with LPS (10μg/ml) or GM-CSF (10 ng/ml) for 24 h and then for a further 24h ± VitD (1μM) or with VitD (1μM) alone for the last 24 h of the experiment. Cell culture supernatants were assessed for elafin (B), hBD2 (D), cathelicidin (F) and SLPI (H) protein expression. Data are expressed as median natural antimicrobial peptide normalised copy number ± interquartile range (box) and range (whiskers). n=6. **P<0.01 compared to control.

Figure 3. Effect of LPS/IL-1β in the presence or absence of 1α,25-dihydroxy1,25-(OH)2_3 (VitD) D/calcipotriol (Cal) on IL-8, TLR4 and VDR mRNA in END1/E6E7 cells. Cells were treated in the presence or absence of LPS (10μg/ml) for 24 h and then for a further 6 h in the presence or absence of VitD. Similarly, cells were treated in the presence or absence of IL-1β (10ng/ml) for 24 h and then for a further 6 h ± VitD/Cal (1μM). Cell lysates were assessed for IL-8 (A-B), TLR-4 (C-D) and VDR (E-F) mRNA expression. Data are expressed as mean normalised copy number ± SEM. n=6. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Host defence peptide mRNA and protein expression in freshly obtained high vaginal and endocervical cells from pregnant women. (A) Host defence peptide gene expression from high vaginal (n=12) and endocervical cells (n=10), (B) Host defence peptide protein expression from high vaginal fluid (HVF, n=12) and endocervical fluid (ECF, n=10), (C) VDR/TLR mRNA expression in HVCs and Data are expressed as mean ± SEM, *P<0.05.

Figure 1


A
Elafin mRNA expression (copy number relative to GAPDH)
control
IL (24h)
VitD (6h)
IL + Vit D (24h + 6h)
Cal (6h)
IL + Cal (24h + 6h)
B
Elafin protein expression (pg/mg protein)
control
IL (48h)
VitD (24h)
IL + Vit D (24h + 24h)
Cal (24h)
IL + Cal (24h + 24h)
C
hBD2 mRNA expression (copy number relative to GAPDH)
control
IL (24h)
VitD (6h)
IL + Vit D (24h + 6h)
Cal (6h)
IL + Cal (24h + 6h)
D
hBD2 protein expression (pg/mg protein)
control
IL (48h)
VitD (24h)
IL + Vit D (24h + 24h)
Cal (24h)
IL + Cal (24h + 24h)
E
Cathelicidin mRNA expression (copy number relative to GAPDH)
control
IL (24h)
VitD (6h)
IL + Vit D (24h + 6h)
Cal (6h)
IL + Cal (24h + 6h)
F
Cathelicidin protein expression (pg/mg protein)
control
IL (48h)
VitD (24h)
IL + Vit D (24h + 24h)
Cal (24h)
IL + Cal (24h + 24h)
G
SLPI mRNA expression (copy number relative to GAPDH)
control
IL (24h)
VitD (6h)
IL + Vit D (24h + 6h)
Cal (6h)
IL + Cal (24h + 6h)
H
SLPI protein expression (pg/mg total cellular protein)
control
IL (48h)
VitD (24h)
IL+VitD (24h+24h)
Cal (24h)
Cal+VitD (24h+24h)

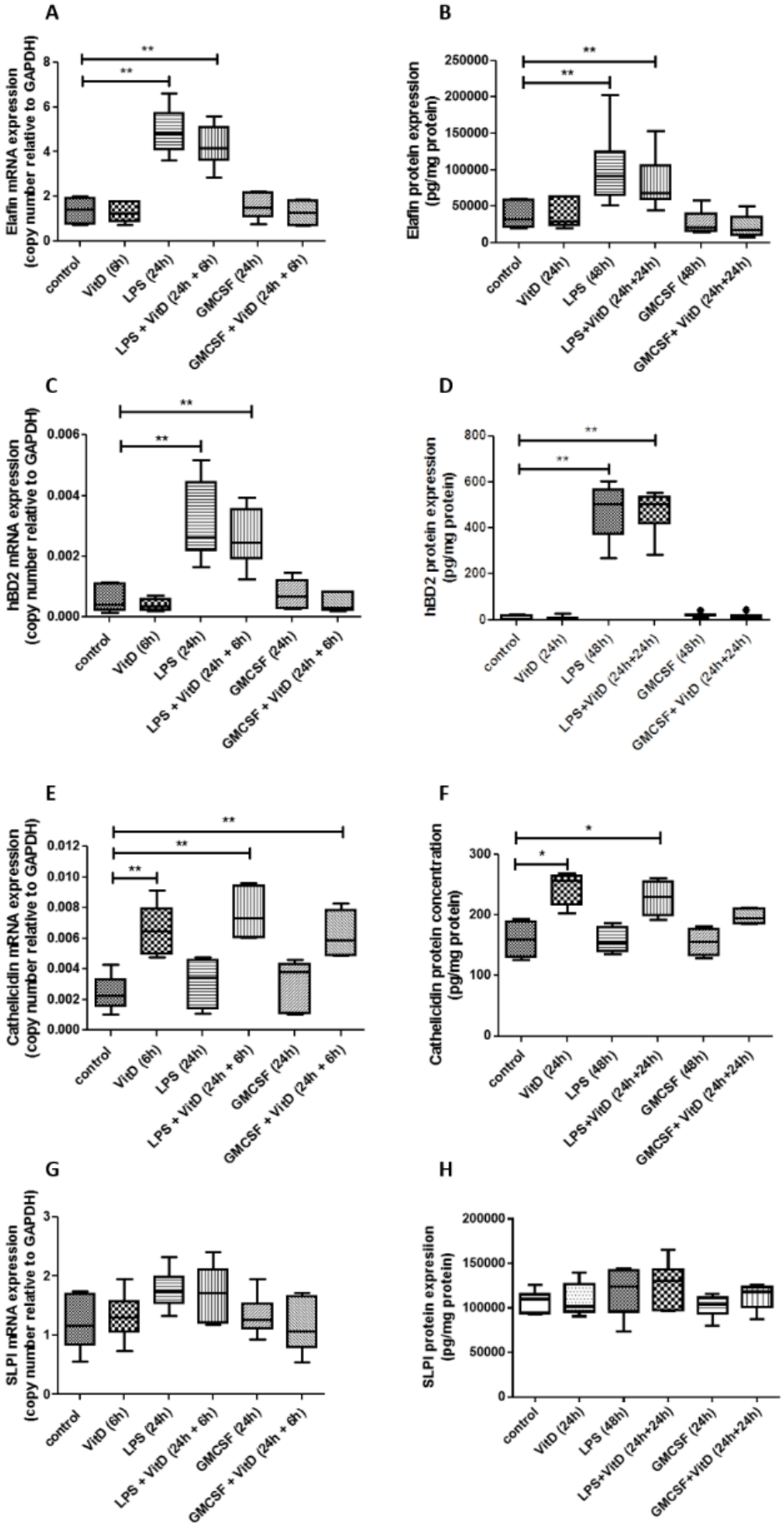

A
Elafin mRNA expression (copy number relative to GAPDH)
control
VitD (6h)
LPS (24h)
LPS + VitD (24h + 6h)
GMCSF (24h)
GMCSF + VitD (24h + 6h)
B
Elafin protein expression (pg/mg protein)
control
VitD (24h)
LPS (48h)
LPS+VitD (24h+24h)
GMCSF (48h)
GMCSF+ VitD (24h+24h)
C
hBD2 mRNA expression (copy number relative to GAPDH)
control
VitD (6h)
LPS (24h)
LPS + VitD (24h + 6h)
GMCSF (24h)
GMCSF + VitD (24h + 6h)
D
hBD2 protein expression (pg/mg protein)
control
VitD (24h)
LPS (48h)
LPS+VitD (24h+24h)
GMCSF (48h)
GMCSF+ VitD (24h+24h)
E
Cathelicidin mRNA expression (copy number relative to GAPDH)
control
VitD (6h)
LPS (24h)
LPS + VitD (24h + 6h)
GMCSF (24h)
GMCSF + VitD (24h + 6h)
F
Cathelicidin protein concentration (pg/mg protein)
control
VitD (24h)
LPS (48h)
LPS+VitD (24h+24h)
GMCSF (48h)
GMCSF+ VitD (24h+24h)
G
SLPI mRNA expression (copy number relative to GAPDH)
control
VitD (6h)
LPS (24h)
LPS + VitD (24h + 6h)
GMCSF (24h)
GMCSF + VitD (24h + 6h)
H
SLPI protein expresiion (pg/mg protein)
control
VitD (24h)
LPS (48h)
LPS+VitD (24h+24h)
GMCSF (24h)
GMCSF+VitD (24h+24h)

Figure 3

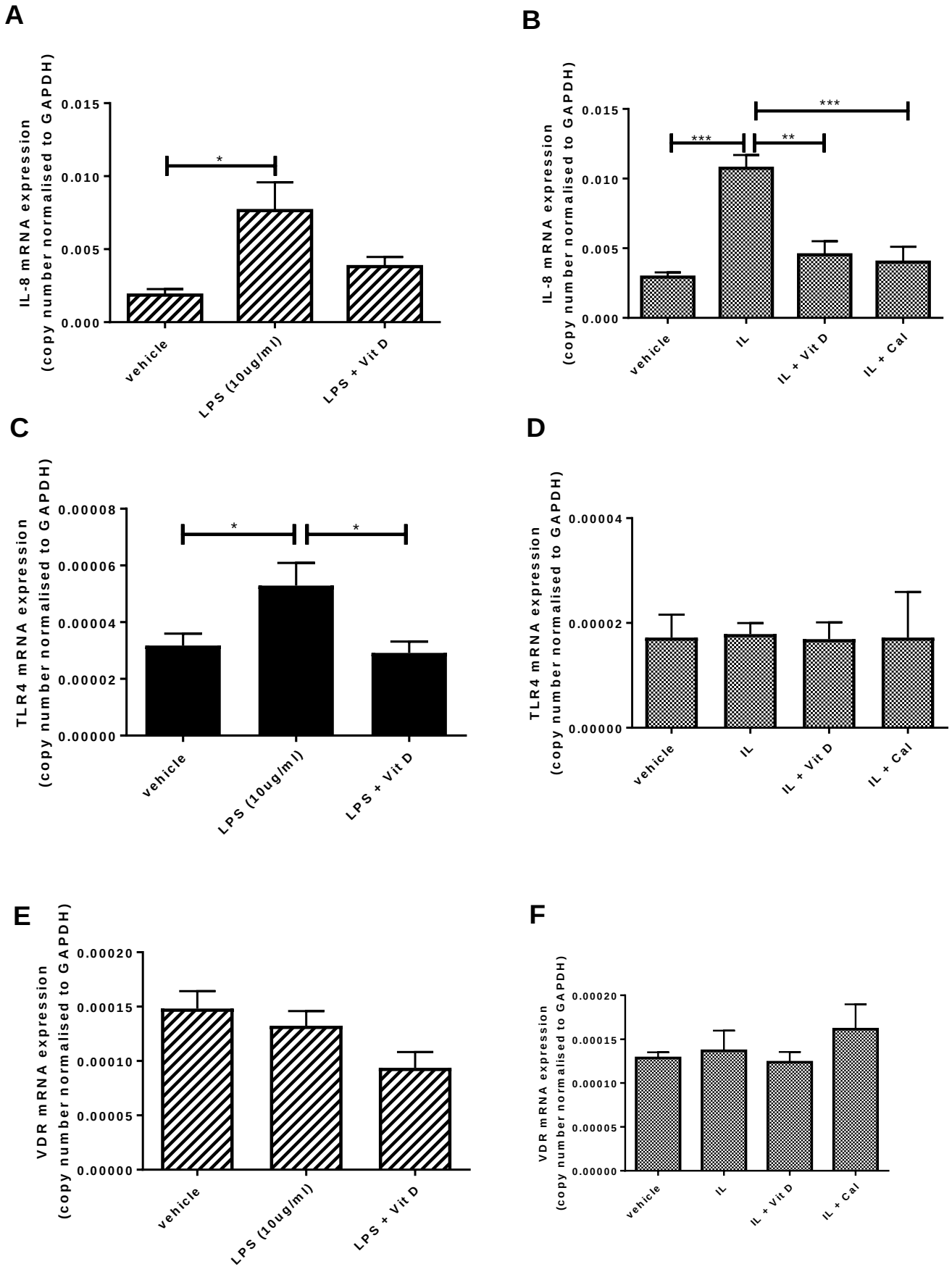
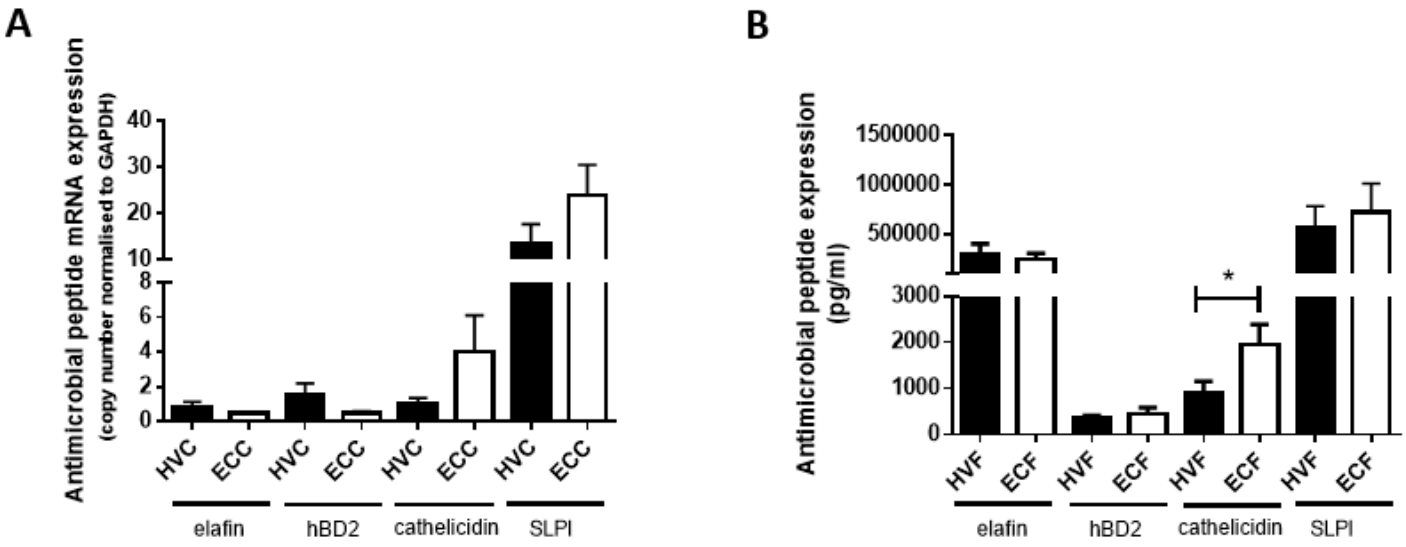
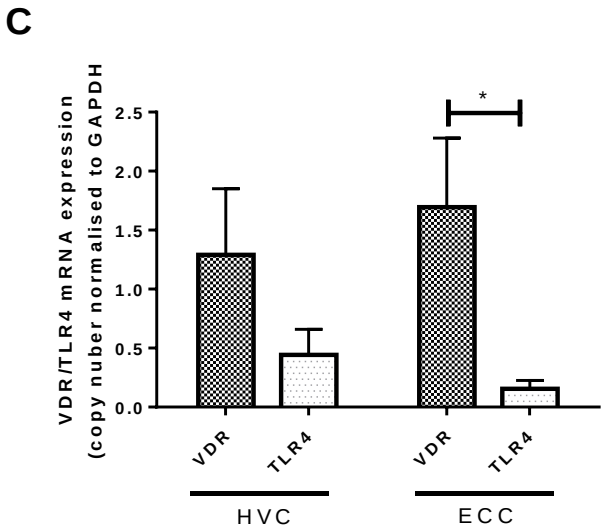

A
IL-8 mRNA expression
(copy number normalised to GAPDH)
0.015
0.010
0.005
0.000
*
vehicle
LPS (10ug/ml)
LPS + Vit D
B
IL-8 mRNA expression
(copy number normalised to GAPDH)
0.015
0.010
0.005
0.000
***
***
**
vehicle
IL
IL + Vit D
IL + Cal
C
TLR4 mRNA expression
(copy number normalised to GAPDH)
0.00008
0.00006
0.00004
0.00002
0.00000
*
*
vehicle
LPS (10ug/ml)
LPS + Vit D
D
TLR4 mRNA expression
(copy number normalised to GAPDH)
0.00004
0.00002
0.00000
vehicle
IL
IL + Vit D
IL + Cal
E
VDR mRNA expression
(copy number normalised to GAPDH)
0.00020
0.00015
0.00010
0.00005
0.00000
vehicle
LPS (10ug/ml)
LPS + Vit D
F
VDR mRNA expression
(copy number normalised to GAPDH)
0.00020
0.00015
0.00010
0.00005
0.00000
vehicle
IL
IL + Vit D
IL + Cal

Figure 4


A
Antimicrobial peptide mRNA expression
(copy number normalised to GAPDH)
40
30
20
10
8
6
4
2
0
HVC
ECC
HVC
ECC
HVC
ECC
HVC
ECC
elafin
hBD2
cathelicidin
SLPI
B
Antimicrobial peptide expression
(pg/ml)
1500000
1000000
500000
3000
2000
1000
0
*
HVF
ECF
HVF
ECF
HVF
ECF
HVF
ECF
elafin
hBD2
cathelicidin
SLPI


C
VDR/TLR4 mRNA expression
(copy nuber normalised to GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
*
VDR
TLR4
VDR
TLR4
HVC
ECC